

# **Single Molecule Techniques for Advanced *In Situ* Hybridization**

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# Single Molecule Techniques for Advanced *in situ* Hybridization

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## **Abstract:**

One of the most significant achievements of modern science is completion of the human genome sequence, completed in the year 2000. Despite this monumental accomplishment, researchers have only begun to understand the relationships between this three-billion-nucleotide genetic code and the regulation and control of gene and protein expression within each of the millions of different types of highly specialized cells. Several methodologies have been developed for the analysis of gene and protein expression *in situ*, yet despite these advancements, the pace of such analyses is extremely limited. Because information regarding the precise timing and location of gene expression is a crucial component in the discovery of new pharmacological agents for the treatment of disease, there is an enormous incentive to develop technologies that accelerate the analytical process. Here we report on the use of plasmon resonant particles as advanced probes for *in situ* hybridization. These probes are used for the detection of low levels of gene-probe response and demonstrate a detection method that enables precise, simultaneous localization within a cell of the points of expression of multiple genes or proteins in a single sample.

There are approximately 30,000 genes in the human genome, equaling about 1 to 1.5 percent of the DNA sequence. [1] In order to determine gene function, individual gene expression has to be identified and linked to the type of cell showing activity. However, individual genes are expressed in specific cells, in specific organs, and at specific times in development. This makes it difficult to identify and characterize all the genes. Typical methods of analysis for the investigation of *in situ* gene expression involve the use of target gene / probe complexes that are used in combination with catalytic chemical and avidin / biotin amplification. This approach provides amplification in the probe location response to that above the background signal due to the native autofluorescence of biological tissue. These amplification procedures limit this approach to only a few targets per tissue section due to limitations in spectrally unique reagents available. Furthermore, they are also hindered from the occurrence of over amplification, which limits confidence in interpretation and quantitative analysis of gene expression. In order to circumvent these problems, our approach involves the use darkfield microscopy in combination with plasmon-resonant silver or gold particles.

Plasmon-resonant particles (PRPs) are nano-sized (50-120 nm) metal particles that have extremely bright light scattering in the visible range. PRPs efficiently scatter light elastically because of a surface plasmon resonance, a collective resonance of the conduction electrons in the metal.[2] These particles scatter narrow bandwidths of light very efficiently and are very bright when illuminated using a white light source through a darkfield microscope (shown schematically as Figure 1A). Each of the bright features in Figure 1B indicates the scattering of single PRP's on the sample surface with an intensity of about million times brighter than single fluorescent molecules. The spectral response, such as peak wavelength, spectral bandwidth, and magnitude, of each particle is determined by many factors including the particle size, shape, material composition, and environment.[3] An example of the spectral dependence on particle dimensions is shown in Figure 2 where single particle scattering spectra are displayed in relation to the predicted particle diameter. This illustrates that the spectral response ranges from the blue to the red for particles with diameters from 50 – 100 nm respectively.

The basic silver colloid production was adopted from a paper by Sheldon Shultz, *et al.*.[3] The process starts with 5 nm colloidal gold spheres in a solution of  $5 \times 10^{13}$

particles/ml. The gold colloids, available from Ted Pella (Redding, CA) are provided in a buffer composed of 20 mM Tris (tris-hydroxymethyl-aminomethane), 20mM sodium azide, 225 mM NaCl, 1% BSA, and 20% glycerol, with a pH of 8.2. The silver enhancement process, similar to photograph developing, uses commercially available chemicals to deposit silver on the surface of the gold spheres. Schultz's original recipe instructed the combination of 20 ml of filtered, double-distilled water with 3  $\mu$ l of 5 nm colloidal gold spheres. Stirring vigorously at room temperature, 2 drops of initiator (~100  $\mu$ l) is added, followed by 60  $\mu$ l of enhancer in 10  $\mu$ l increments. Under these conditions, the solution completes its development process in 1 or 2 minutes and appears translucent yellow in transmitted light. The PRP solutions were characterized by depositing PRP samples on Mica substrates with subsequent imaging using the dark-field microscope. Mica substrates were used in place of glass slides because they have less background scattering from debris and surface scratches.

There are many variables in the silver enhancement process. In an attempt to develop monodisperse populations for use in a multiplexed analysis, many of these variables were manipulated. Of most interest and focus were particle growth time, particle density, temperature and variations in chemical ratio. As shown in Figure 3, the adjustment of these growth conditions was successful in yielding 3 monochromatic distributions of particles ranging from the blue to the red. However, these results were difficult to reproduce and the development of a robust procedure was not discovered.

In lieu of reproducibly manufactured monochromatic distributions a single target *in situ* PRP hybridization analysis could be demonstrated using a polydispersed solution of particles. The functionalized PRPs were developed using the silver enhancement process described previously with exception to the 5 nm colloidal gold starting material, which was purchased functionalized with a anti-biotin antibody (Nanoprobes, Stony Brook, New York). The results described in this report and previous research, mainly for electron microscopy applications, indicate that the silver enhancement of the immunolabeled gold particles does not degrade the biological activity of the surface protein.[3, 4] Figure 4 illustrates the attachment of the functionalized PRPs to a biotinylated probe designed to detect a specific mRNA expression corresponding to a

specific gene expression. Therefore, the presence of a PRP in a given region or cell type of the tissue sample indicates gene expression.

The tissue samples were prepared the same way as if they were to be used for fluorescence ISH (FISH). Lisa Stubbs' mouse genomics group at Lawrence Livermore National Laboratory provided the mouse embryo tissue samples for the experiment.[5] *In-situ* hybridization (ISH) is a routinely used method of identifying gene expression in specific cells. The technique involves slicing 10-micrometer-thick sections of tissue (about the thickness of a single cell) to place on a glass slide for viewing of single cells in their native environment allowing organs and cells to be identified. A mouse fetus is only a couple centimeters long, allowing a whole body slice to be analyzed on one slide. A gene probe, which will attach to the unique RNA sequence produced by the gene under study, is added to the tissue slice. When the tissue is imaged with conventional microscopy, the probes can be observed binding to and highlighting the cells in which the particular RNA has been expressed.

For the purposes of this research the PEG3 Gene was used to validate this method since it has been well characterized through previous research.[6] This gene is common to both the genomes of mice and humans. This is of importance in that if mice are to be used as human models for biomedical research, the human and mouse genomes need to be compared. It has been found to affect embryonic mouse development and mouse maternal behavior. Research shows that a removal of the PEG3 gene in mice results in mothers ignoring their babies to the point that the babies die. A similar protein is expressed in the human brain, which may show that maternal instinct was conserved through evolution. During the embryonic mouse development, there is also a high expression in the skin cells, (Parker, 2001) which we explored as to validate the PRP ISH method.

We had successful results with our PRP ISH validation. Figure 5A is a representative darkfield microscopy image of a mouse tissue section in a skin region, which was treated with the biotinylated PEG3 gene probe. As expected, the functionalized PRPs were clearly found in the skin tissue showing primarily blue scattered light. The surrounding tissue shows a less intense white response due to background scatter from unlabeled tissue, indicating a lack of specific PRP attachment.

Very little non-specific binding can be seen, where PRPs have been lodged in the tissue or attached to the Mica but do not necessarily identify a target location. Figure 5B and C shows segments of mouse embryo skin tissue that was not treated with the biotinylated probe prior to the addition of the functionalized PRPs. Few of the antibiotin labeled PRP's have attached to the sample in comparison to the response observed in Figure 5A.

The results presented here demonstrate the capability of advanced *in situ* hybridization methods based on the use of PRP and darkfield microscopy. The successful analysis in mouse tissue sections was demonstrated without the need for amplification steps. This result indicates a significant improvement compared to standard approaches requiring amplification. Future work will provide a robust fabrication protocol capable of producing multiple monochromatic distributions of PRP enabling multiplexed PRP-ISH.

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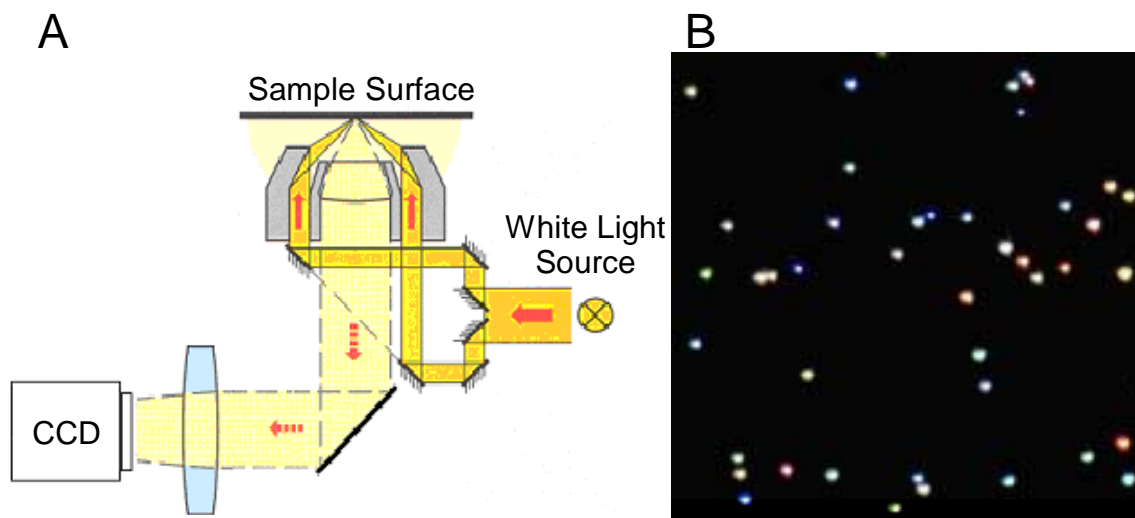


Figure 1: (A) Schematic of a darkfield microscope. (B) Darkfield microscopy image of PRPs dispersed onto a mica substrate.

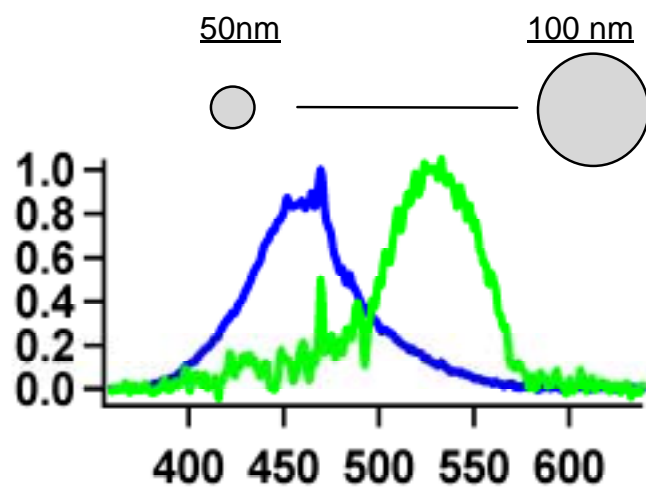


Figure 2: Single particle scattering spectra of two representative PRPs shown in relation to the predicted size.

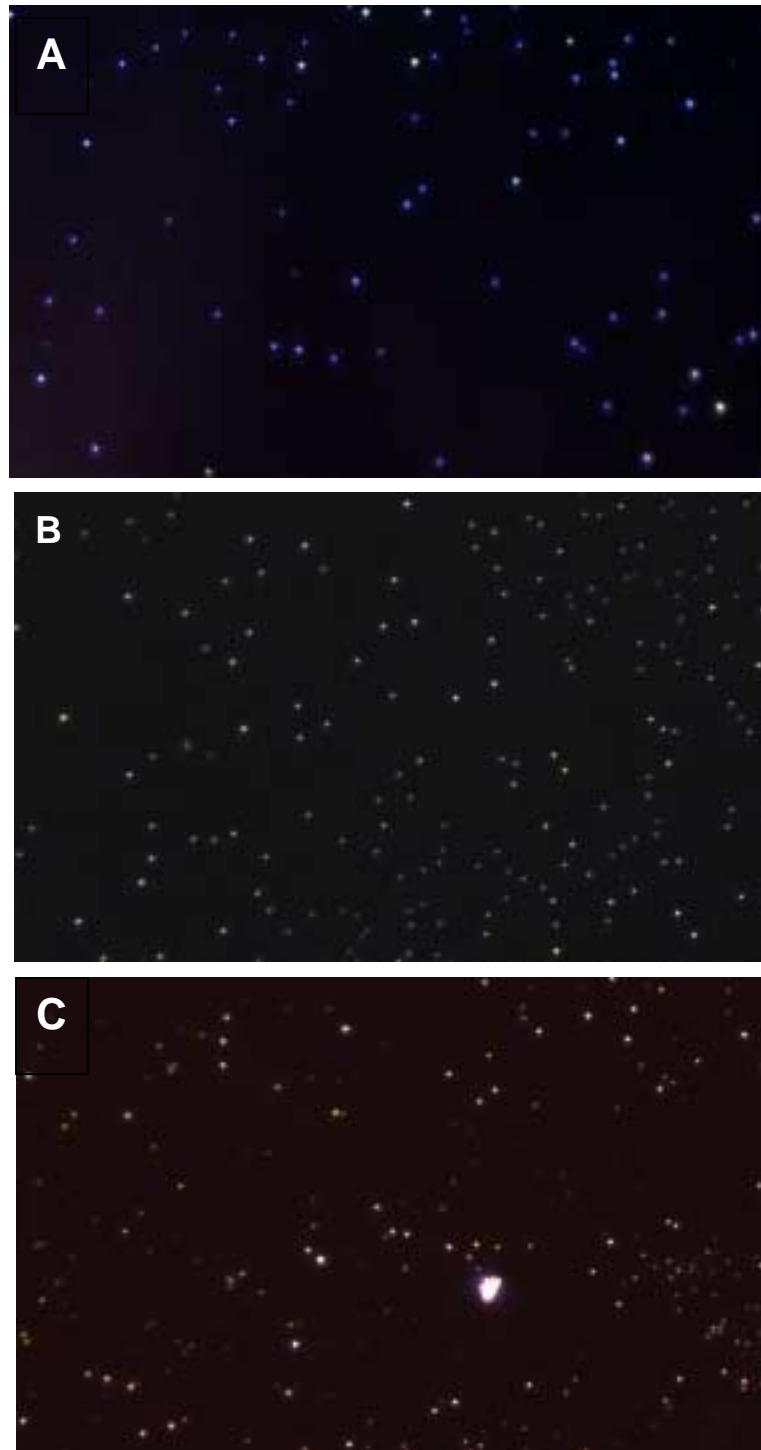


Figure 3: Darkfield images of single PRPs on mica substrates. These preparations illustrate 3 monochromatic distributions consisting of primarily (A) blue, (B) green, and (C) red scattering characteristics.

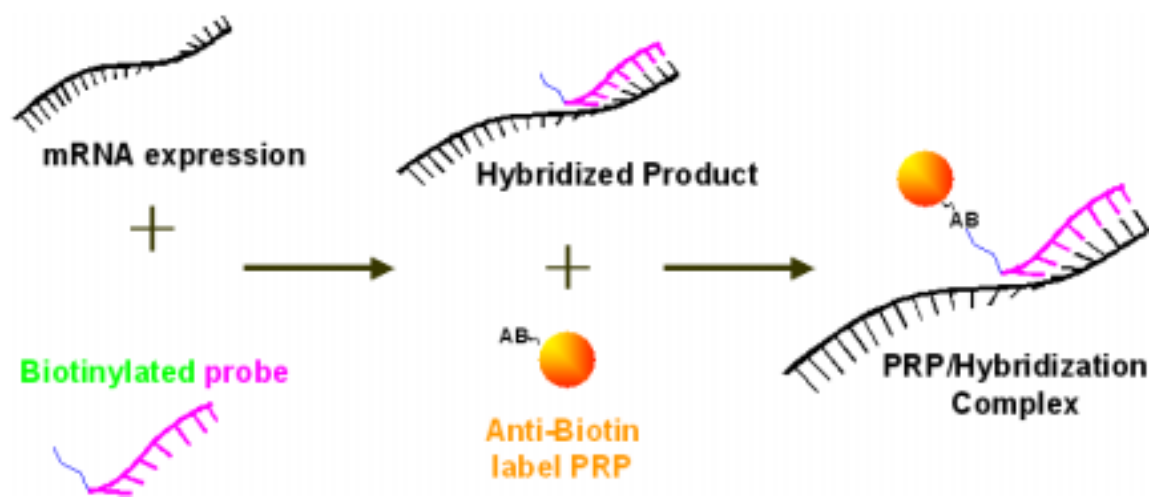


Figure 4: Labeling scheme for investigating gene expression using PRPs in tissue sections.

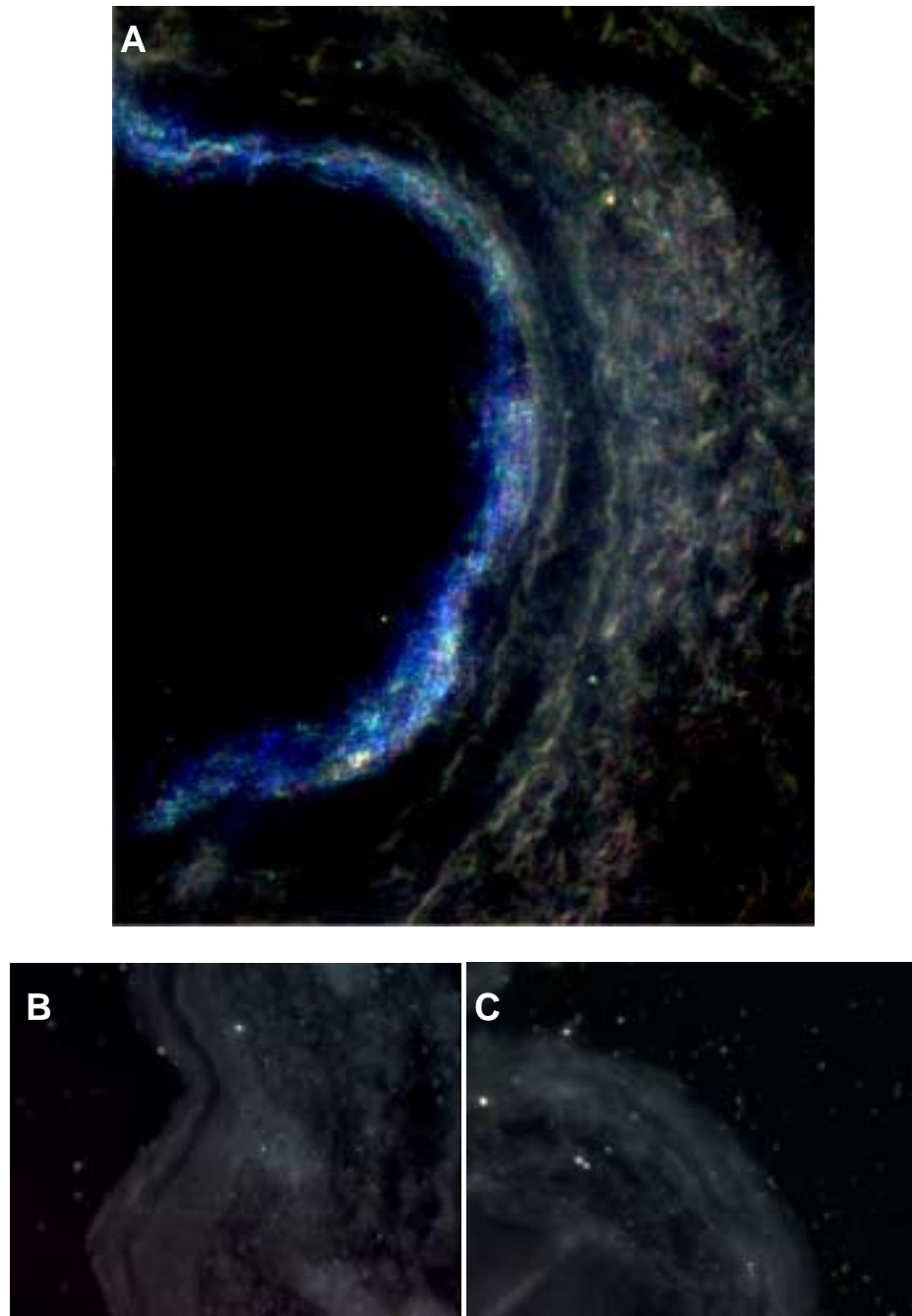


Figure 5: Darkfield images of mouse tissue sections. (A) Tissue treated with the biotinylated probe. The blue scattering region indicates expression of the PEG3 gene. (B) and (C) Tissue that lacks the probe.